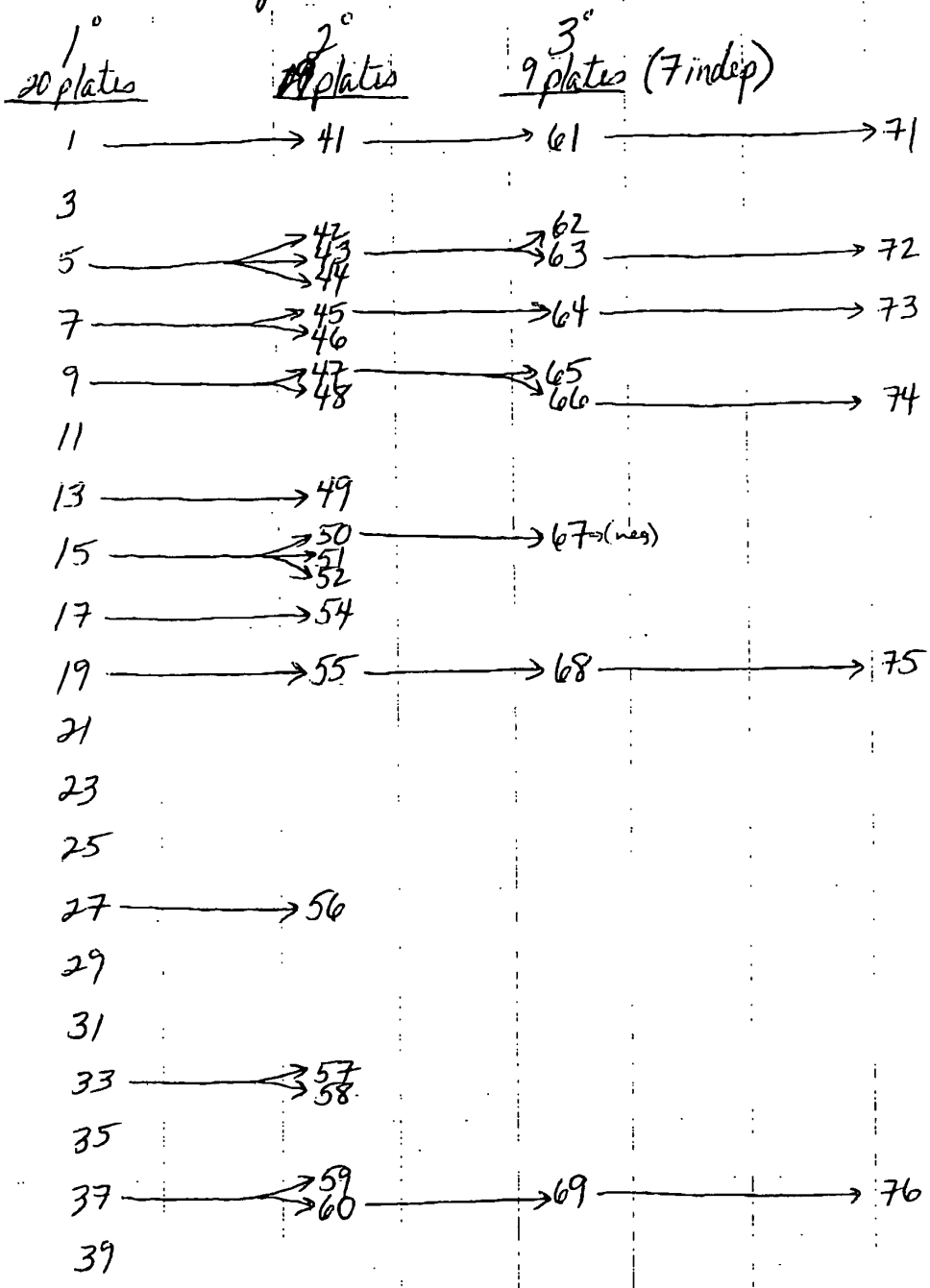
Exhibit 9

Lab Notebook Pages of
Dr. Susan L. Acton

Successive Rounds of screening to clone the murine SR-BI from a 3T3-L1 Adipocyte pcDNAI Library



Probe: 500-600 bp BamHI fragment (5') of huSR-BI in pcDNAI

3T3-L1 Adipocyte Library

pCDNA1 in MC1061/P3

G. Balding electroporated + plated bacp, then scraped plates + froze. This is what she gave me. (3 vials)

Use vial 2

Scrape a little + thaw, dilute 1:100, then 1:50 and plate 300 µl on a 10 cm plate

She did 20 plates of 30,000 colonies (must have been very small colonies)

Note that the library is non-directional + is in the BstX I sites in pcDNA1

XJ58 Screen 3T3-L1 Adipocyte Library for murine SR-BI

Purpose: Clone the murine SR-BI for further analysis

Plate Library

Pour 100mm LB Amp/Tet (15/8 μg/ml) Plates
Let dry 48 hrs r.t.

Scrape some bugs from frozen tube 2 (from G. Baldini Lodish lab) of 3T3-L1 library in MC1061/P3 pGDNA1 expression)
Thaw scraped bugs, dilute 1:100, then 1:17 and plate 50μl

2 μl undil bugs tube #2
198 μl LB

200 μl of 1:100
↳ 118 μl of 1:100
1.882 ml of LB

2 ml of 1:17
↳ plate 50μl/100mm plate

Incubate plates 14 hrs (6pm-8am) 37°C
Transfer plates to 4°C 1hr

Make Lifts

① Carefully lay down sterile Nitrocellulose Millipore HATF prenumbered, number side down onto bugs
② When wet, poke 3 holes thru membrane and plate
③ Lift filter off plate with blunt end forceps

~~Lay~~

④ Lay bug side up onto fresh LB A/T plate
⑤ Lay another filter (pre-numbered) numby-side down onto bugs on filter to make sandwich
⑥ Poke holes thru duplicate filter to match holes in original filter
⑦ Let bugs grow 3 hrs 37°C between filters
⑧ Let bugs regrow on original ~~filter~~ plate 3 hrs - then store wrapped in parafilm 4°C.
⑨ As in Maniatis, (1.98) lyse colonies
Set up 4 trays with whatman 3MM Paper soaked with (in order)

① 10% SDS
② Denaturing soln (0.5N NaOH, 1.5M NaCl)
③ Neutralizing soln (1.5M NaCl, 0.5M Tris-Cl pH 7.4)
④ 2X SSC

a) Peel nitrocellulose sandwich from gel & place on SDS-impregnated 3MM paper 2-3 min
b) Wipe off excess with edge of tray & transfer to denat. tray 5 min
c) Transfer to Neutral tray 5 min.
d) Transfer to 2X SSC tray ~~5 min~~ but separate sandwich first - 5 min
i) Transfer to dry 3MM paper colony side up Dry at least 30 min r.t.

⑩ Sandwich filters between 2 sheets of dry 3MM paper Fix by baking, in 80°C in vacuum oven
⑪ Store filters under vacuum no heat

X158 cont'd

Make SR-BI probe

Digest #		10X AcBSA	H_2O	10X NEB Buffer	enzyme
① phaSRIII (1.03 µg/µl)	10µl	5µl	28µl	5µl #2	PstI / HindIII 1µl / 1µl ↓
② pcDNA1 (1 µg/µl)	10µl	5µl	28µl	5µl #2	
③ phaSRIII	10µl	5µl	28µl	5µl BamHI buffer	BamHI 2µl ↓
④ pcDNA1	10µl	5µl	28µl	5µl	

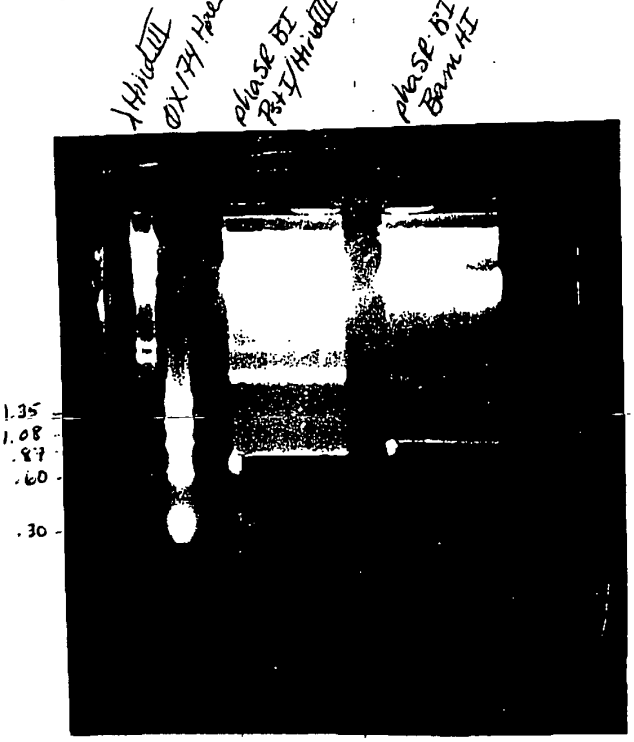
Incubate 37°C 2hr 2:10-4:10 freeze digest
Ran mini-gel; all digests looked good.
Clean up digests ① + ③

Phenol extract (50µl)
Back extract w/ 50µl TE
Precip DNA in 100µl
with 10µl 3M NaOAc
260µl 100% EtOH
-80°C 20 min
Spin 20 min 4°C
Wash w/ 70% EtOH (cold)
Speed Vac Dry
Resuspend in 18µl TE
Add 6µl 10X blue juice
Heat 68°C 10 min

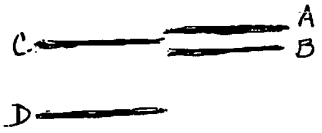
Run on 1% Low melt (Seaplaque) TAE gel 4°C overnight 6V turned up to 20V at noon Cut out band at 6 pm

λ HindIII ØX174 AseIII Digest ① 4 lanes Digest ③ 4 lanes

X458 cont'd



Probes



For screening lifts, use probe B

Label Probe

① Melt probe B, take 10µl & add to 10µl H_2O. Label as probe B diluted 1:1

② Combine 5µl diluted probe B
5µl primers (from Amersham kit 9mers)
Boil 5'
spin down liquid

③ Add 10µl labeling buffer (kit)
23µl H_2O
2µl enzyme
5µl $\alpha^{32}P$ dCTP
Mix by pipetting gently
Spin
Put in 37°C warm room ~~45 min~~ 2hr 2pm - 4pm

X158 cont'd

Check incorporation

Combine
20 µl salmon sperm DNA
<1 µl labeled rxn
180 µl 5% cold TCA

Spin 10' 4°C
Remove 100 µl & count as top
Count remainder as bottom

% incorp = $\frac{\text{bottom} - \text{top}}{\text{bottom} + \text{top}} \times 100\% = 22\%$

% incorp = 22%

Clean up probe:
Add 5 µl LPA to rxn
25 µl 7.5 M NH4 Acetate
125 µl 100% EtOH (cold)
Put at -80°C 20 min
Spin 20 min 4°C
Rinse w/ cold 70% EtOH
Speed-Vac dry
Resuspend in 30 µl TE
Add to prehyb soln containing filters
Hybridize 50°C overnight

X158 cont'd

Prehyb - 50°C

500 mM PB, 1mM EDTA, 7% SDS, 1% BSA, 100 µg/ml salmon sperm DNA (boiled prior to adding)

For 60 mls: 30 mls 1M PB ✓
120 µl 500 mM EDTA ✓
21 mls 20% SDS ✓
0.6g BSA ✓
0.6 ml salmon sperm DNA ✓
8.28 ml H_2O ✓

Hyb - 50°C in prehyb soln + probe

Washes -

Wash blot 2X fast w/300mM PB r.t.
wash " 1X 10' r.t.
Wash " 2X 10' Soln A (300mM) 54°C
Wash " " " B (300mM) 54°C

Put down on film - 48 hrs r.t.
a) Let filters air dry on whatman
b) Arrange on old piece film covered with saran wrap
c) ~~Cover~~ & Tape down filters & cover w/saran wrap
d) Put markers (from Stratagene ruler) down to orient film w/ filters

Results:

> 100 small dots which are on duplicate lifts. Background is low. Looks good!

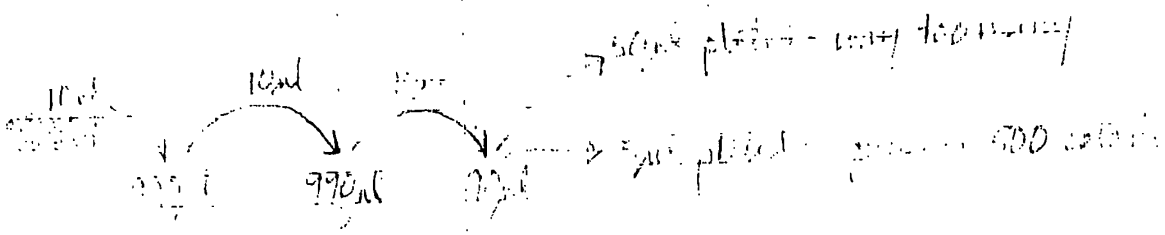
X158 cont'd

Secondary Lifts

A. Picked 19 positive dots, 1 negative control from primary lifts

B. Used pasteur pipets, large end to pick plugs from plates into 0.5 ml LB + Amp/Tet Overnight 37°C

C. Make dilutions



D. Plate bugs
First time plated way too many so replated ~13 hrs 37°C

E. Transfer plates to cold room 1 hr 4°C

Lifts

① Carefully lay down sterile HATF nitrocellulose Millipore prenumbered, number side down onto bugs

② When wet poke 3 holes thru membrane and plate

③ Lift filter off plate with blunt end forceps

④ Lay bug side up onto fresh LB A/T plate (LB Kan for 54-60)

⑤ Lay another filter (labeled B) number side down onto bugs on filter to make a sandwich

⑥ Poke holes thru duplicate filter to match holes in original filter

(7) Let bugs grow 3 hrs 37°C between filters

(8) Let bugs grow on original plate 3 hrs then store wrapped in parafilm 4°C

(9) As in Maniatis (1.98) Lyse colonies
Set up 4 trays with Whatman 3MM paper soaked with

3 min	(1) 10% SDS
5 min	(2) Denaturing soln (0.5N NaOH, 1.5M NaCl)
5 min	(3) Neutralizing soln (1.5M NaCl, 0.5M Tris Cl, pH 7.4)
5 min	(4) 2X SSC

a) Peel nitroc. sandwich from gel + place on SDS-impreg. 3MM paper
b) Wipe off excess with edge of tray + transfer to denat. tray 5 min
c) Transfer to neutral tray 5 min
d) Transfer to 2X SSC tray after separating filters
e) Transfer to dry 3MM paper colony side up. Dry at least 30 min r.t.

(10) Sandwich filters between sheets of dry 3MM paper. Fix by baking 1 hr 80°C in vacuum oven

(11) Store filters under vacuum no heat

Label probe (5' BamHI fragment of haSR-BI) as for 1° lifts

ID:CERENKOV PRESET TIME: 1.00 TUI
SAMPLE REPEAT: 1 CYCLE REPEAT: 1 BCR:N RS232:N
CHANNEL 1 ... SIGMA: 2.00 BKG ... 0.00 BKG 2SIG: 0.00 LSR:
CHANNEL 2-LL:600 UL:1000 2SIGMA: 2.00 BKG SUB: 0.00 BKG 2SIG: 0.00 LSR:
DATA CALC: CPM, UNKNOWN REPLICATES: 1 NORM FACTOR:01.00000
HALF LIFE(DAYS):N

SAM	CPM1	CPM2	TIME
1	[illegible]36.54	757.00	1.00
2	[illegible].97	324.00	1.00

36% incorporation

[illegible] ~~[illegible]~~ 6 ok

Wash blots - Note: May have accidentally used 600mM PB final rather than 300mM

① Wash blot 1X fast w/ 300 mM PB r.t.
② Wash blot 1X 10' r.t. 300 mM PB
③ Wash 2X 54°C soln A 300 mM 10' each

Wash A: 300 mM PB, 5% SDS, 0.5% BSA, 1mM EDTA

For 500 ml: 300 ml 0.5M PB
25 g SDS
2.5 g BSA
1 ml 500 mM EDTA
H_2O to 500 ml

④ Wash 2X 54°C soln B (300 mM PB) 10' each

Wash B: 300 mM PB, 1% SDS, 1mM EDTA

300 ml 0.5M PB
15 ml 20% SDS
1 ml 500 mM EDTA
H_2O to 500 ml

Dry & expose to film

① Let filters dry on whatman paper
② Use old piece of film as support
wrap it in saran wrap
put filters on it & tape down
put stratagene ruler pieces on it to line up w/ 1° & 2° filters
cover with saran wrap
③ Expose to film 2 days

Results: 2° lifts don't look as good as 1° - should not have amplified plug pick from 1°. I think high background may be due to accidental use of 600 mM PB rather than 300 mM PB.

Still there seem to be some definite positives so picked them for 3°.

X158 cont'd

3° Lifts

A. Picked 9 positive dots (#61-69), negative control (#70) from secondary lifts

B. Used sterile pasteur pipets, large end to pick plugs into 0.5 ml LB. Vortexed well.

LB
plug + 500 µl

		results
10 µl + 990 µl	→ plate 50 µl	way too many
10 µl + 990 µl	→ plate 50 µl	way too many
100 µl + 900 µl	→ plate 50 µl	just about right ~200/plate

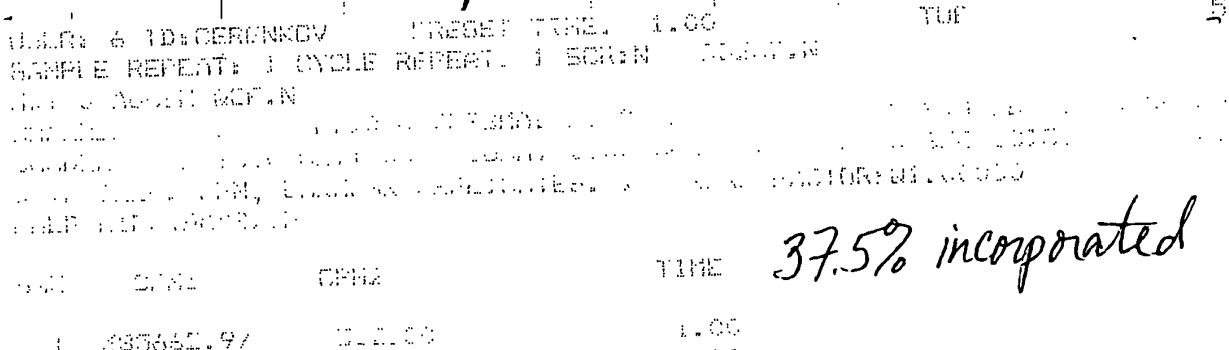
Grow ~14 hrs 37°C

C. Transfered highest dilution plate to 4°C for lifts

D. Performed lifts as before

Made mistake of reincubating master plates for ~48 hrs rather than ~6-8 hrs. Plates dried somewhat and colonies grew big

E. Label probe as before



37.5% incorporated

Prehyb } as before 30°C, 300mM PB
Hyb }

Wash 3° as before - 54°C, 300mM
...on Put down on film - w/screen

X160

Plasmid midiprep

preps: 6 single isolates from 3° screen #71-76

Day 1

1. Pick a single colony into 25 mls LB Amp/Tet and grow overnight shaking 37°C.

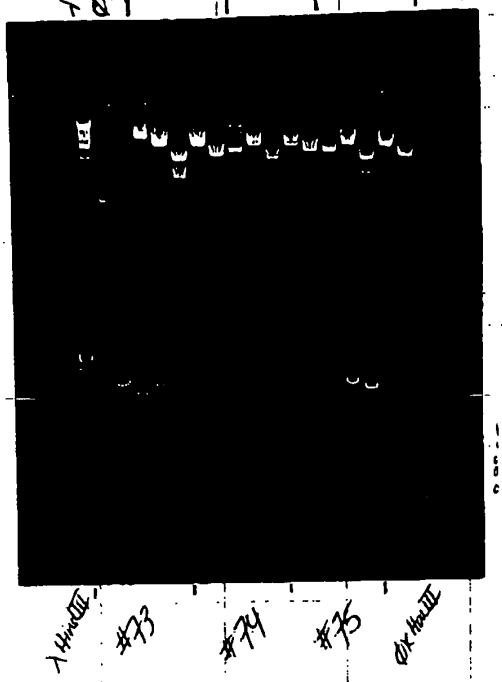
Day 2

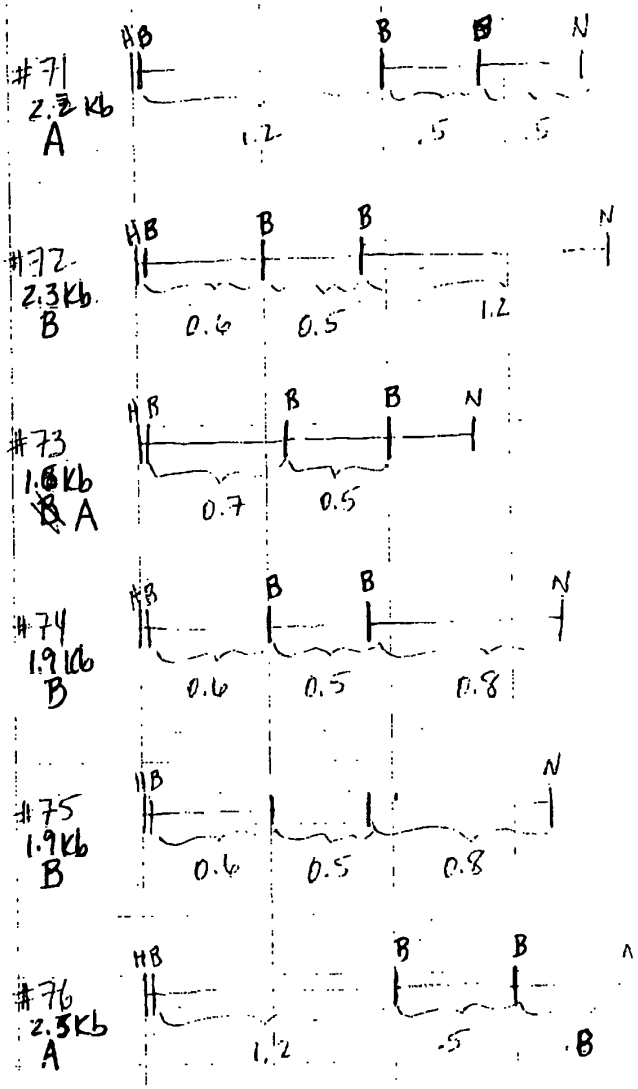
2. Take 400 µl out into freezer vial, add 100 µl glycerol and freeze at -150C.
3. Transfer ~~remainder~~ to Falcon 2059 15 ml tube on ice.
4. Spin ~~rest~~ in SS-34, 9000 rpm, 2 min 4C. Add remaining half.
5. Dry pellet as much as possible.
6. Resuspend pellet in 500 µl ice cold solution I by vigorous vortexing.
7. Add 1 ml fresh solution II (0.2 N NaOH, 1% SDS)
   for 100 ml: 1 ml 2 N NaOH
   0.5 ml 20% SDS
   8.5 mls ddH20
   Swirl gently until clear. Do not vortex. Leave on ice 10 min.
8. Add750 µl solution III (ice-cold). Close tube and mix contents by shaking vigorously several times. Store on ice 5'. A flocculent white precipitate should form.
9. Centrifuge 15', 4°C, 9000 rpm.
10. Recover supe and add equal volume of phenol:chloroform. Mix by vortexing.
11. Spin 9000 rpm, 5'.
12. Add 2 volumes, ethanol r.t., vortex, let stand 5'.
    Spin in SS-34 rotor for 15' 9,000 rpm.
13. Carefully aspirate off all liquid (want it dry) and rinse once in cold 70% EtOH. Aspirate again getting any extraneous drops. Airdry 10' minutes.
14. Redissolve in 100 µl TE ~~plus DNAse-free RNAse (20 µg/ml). Vortex briefly. Incubate 37C, 0.5 - 2 hr. Transfer to sterile eppendorf.~~ Refrig 4°C o.N.
15. ~~Quantitate by dilutions onto EtBr plate.~~
    Add DNAse free RNAse to final 10µg/ml

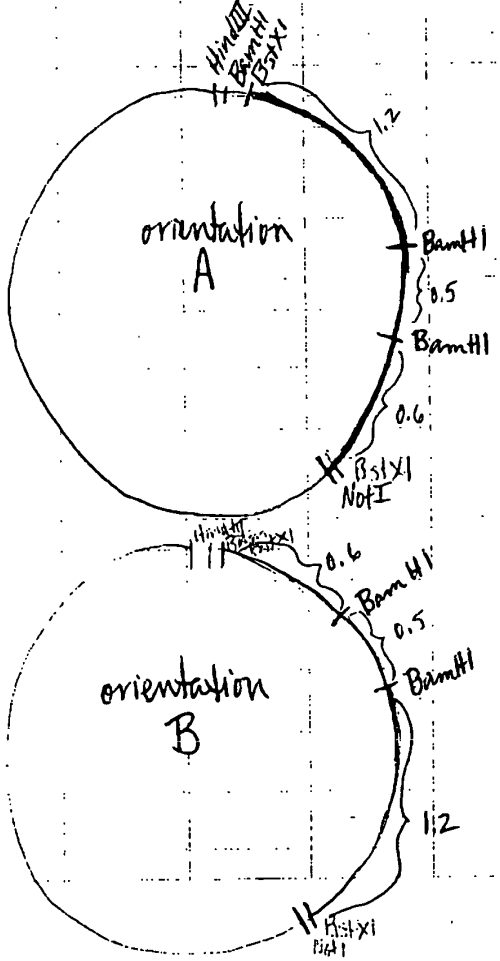
X160 cont'd Digests of Midi-preps

Digest #	DNA	10X AcBSA ul	NEB Buffer 10X	H_2O	1ul each enzymes	~~DNA~~ Fill&Kalf
1	#71 - 2 ul	2 ul	2 2ul	14 ul	–	0.5 ul
2			2	13	Hind III	
3			2	12	Hind III / Not I	
4			2	13	Pst I	
5			u	13	Bam H I	
6	#72					
7						
8						
9						
10						
11	#73					
12						
13						
14						
15						
16	#74					
17						
18						
19						
20						
21	#75					
22						
23						
24						
25						
26	#76					
27						
28						
29						
30						
	37°C	11:45 am - 2:45				

X160 cont'd







Note that orientation B was found to be correct after sequencing of #74 + #76

X1161

DEAE dextran transfections of COS M6 cells

materials:

1. 35 mm dishes
2. DMEM with 10% FBS
3. Chloroquine (40 mM in CMF PBS, sterile filtered)
4. DNA
5. CMF PBS
6. DEAE-dextran (10 mg/ml in CMF PBS (autoclaved))
7. DMSO
8. cPBS
9. sterile tips

method:

day 0 (set up cells)

Set COS M6 cells in 100 mm dishes

(Set 1 confluent T75 into 2.25 100 mm dishes; or 1.5 x 10^6 cells/dish in 10 ml DMEM with 10% FBS

day 1 (transfect)

1. In sterile polypropylene tubes prepare for each dish add (in order):
   a) add CMF PBS to 1.9 ml
   b) DNA - 18 µg/dish
   c) 100 µl of 10 mg/ml DEAE-dextran, vortex well (7 seconds)

tube	# plates	DNA			CMF PBS	10 mg/ml DEAE-dextran
1	1	pheSR-BI (1.	1.03 mg/ml	9.7 µl	1.9 ml	100 µl
2	1	#74 (7/22/94)	1.94 mg/ml	5.2 µl	1.9 ml	100 µl
3	1	#76 (7/22/94)	0.85 mg/ml	11.8 µl	1.9 ml	100 µl
4						

2. Rinse cells with 10 mls CMF PBS (37C). Aspirate PBS.
3. Add transfection cocktail from step 1 and distribute evenly. Incubate 30 minutes, distributing liquid every 10 min.
4. Add 8 ml DMEM 10% FBS + 80 µM chloroquine and incubate 37C 2.5 hrs.
5. Aspirate off medium and replace with 5 ml 10% DMSO in DMEM 10% FBS for 2.5 min.
6. Aspirate off and wash once with 10 mls cPBS.
7. Refeed with 10 ml warm DMEM 10% FBS/dish. Incubate overnight.

X161 cont'd

Lipoprotein 4°C Binding Assay

materials:
1. 6-well dishes
2. DMEM with 10% FBS
3. iodinated and unlabeled lipoprotein
4. Tris-HCl wash buffer
5. Tris-HCl BSA wash buffer
6. 0.1 N NaOH
7. 10 x 75 tubes

method:

day 0: Set up cells

Set transfected COS in 6-well dishes at 1 x 10^6 cells/well in 3 ml/well DMEM + 10% FBS + 1 mM Nabutyrate.

day 1: Binding assay

1. Cool cells down on ice for 30 minutes.
2. Refeed cells labeled ligand 1 ml/well (Hams + HEPES + ~~10% FBS~~ 5% NCLPDS).
   2 Hot - ^{125}I-lipoprotein: 10 µg/ml ^{125}I AcLDL (#60 10.4 mg/ml) LDL #161B (39 mg/ml)
   1 Hot + cold: + 4 µg/ml M BSA #230 DR (3.2 mg/ml)
   Incubate 2 hrs 4°C (in cold room) on shaker.
3. Wash cells (1 ml each)
   3X fast with BSA wash buffer
   2X 5 min with BSA wash buffer
   2X fast with Tris wash buffer
4. Add 1.0 ml 0.1 N NaOH. Leave 20 min r.t. on shaker
5. Remove 50 µl to 10 x 75 tube and freeze for Lowry.
6. Count 500 µl of rest of sample.
7. Count 10 µl of medium + label for specific activity.

For 14.5 ml hot need: 14.5 ml Hams + HEPES + 5% NCLPDS + 145 µl ^{125}I-AcLDL (#61)

5 ml + 51.3 µl LDL #161B

3 ml + 375 µl M-BSA #230

#74

−	+ LDL	+ M-BSA
1	3	5
2	4	

#76

6	8	10
7	9	

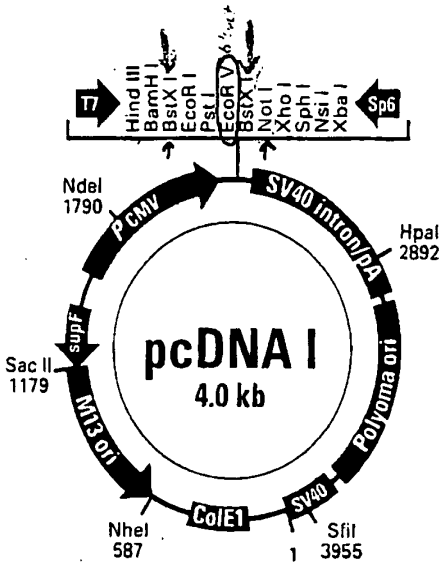
hSR-BI

11	12	13
		14

X163 Prepare blunt-cut pcDNA1 for reversing orientation of clone #76 mSR-BI

Purpose: Reverse orientation of clone #76 which appears to be full-length mSR-BI

Approach: Cut mSR-BI out of clone #76 with HindIII/NotI, chew ends to blunt it, ligate into blunt & CIPed pcDNA1



Cut pcDNA1 with EcoRV which is a blunt cutter

DNA	H_2O	10X AcBSA	10X NEB Buff 2	EcoRV 20,000 u/ml
~~#76 (0.5 µg/µl)~~ 15.5 µl (= 20 µg) pcDNA1 (1.29 µg/µl) 10/29/92	14.5 µl	4 µl	4 µl	2 µl

Incubate 37°C 12:50 pm - 12:50 pm (Thurs)

← after 3 hrs

Add 60 µl dd H_2O, extract w/ 100 µl phenol:chloroform:isoamyl alcohol 25:24:1
Add 10 µl 3M NaOAc pH-5, add 250 µl 100% EtOH, Freeze -20°C 1 hr
Spin 10 min 4°C

[X163] cont'd

4 blunt-cut pcDNA1 (cont'd) - CIP

redissolve in 90µl 10 mM Tris·Cl (pH8)
remove 2µl & save as unCIPed

To remainder add 10µl 10X CIP dephosph. buffer and 8 Units of CIP:

for blunt 1U/2pmoles, estimate 20µg = 16 pmoles
so 8 Units CIP

Incubate 15 min at 37°C 3:35 - 3:50

Then ~~add 8 Units and~~ incubate 45 min at 55°C. 3:50 - 4:35

Kill the CIP

Add SDS + EDTA (pH8.0) to final 0.5% & 5mM, respect. (5µl 10% SDS, 1µl 0.5M EDTA)
Mix well
Add proteinase K to final 100µg/ml (10µl of 10 mg/ml)
Incubate 30 min 56°C. (4:50 - 5:20pm)

Cool the rxn to r.t.
Extract once w/phenol, once w/phenol:chloroform
Add 1/10 vol 3M NaOAc pH ~~3.0~~ 5.0 (10µl)
Mix well add 2.5 EtOH (250µl)
Mix & store @ -20°C ~~o/n~~ 2½hrs
Centrifuge
Wash pellet w/70% EtOH
Redissolve in TE

Ran overnight on 1% Nusieve (should have been Seaplaque) Agarose gel. Band was wary, but cut it out anyway

X164 Prepare #76 mSR-BI cDNA for reinserting into pcDNA1 in correct orientation

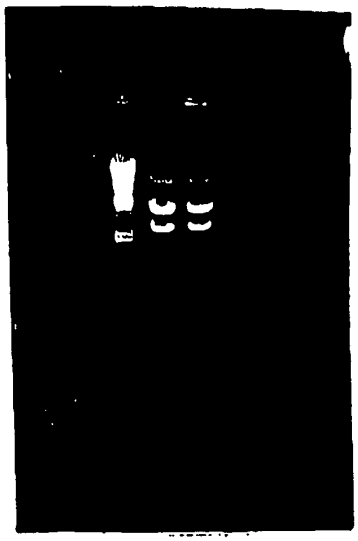
Purpose: Cut out #76 insert to be reinserted in sense orientation. #76 plasmid appears to be full-length mSR-BI but is in the incorrect orientation in pcDNA1.

Digest #76 plasmid:

	DNA	H_2O	10X AcBSA	10X buffer	HindIII	NotI
(1)	#76 (0.85 µg/µl) 24µl (= 20µg)	46µl	10	10 (NotI)	5	5
(2)	#76 24µl	46µl	10	10µl (NEB 2)	5	5

5:45 pm 8:15 pm 37°C

Remove 2µl from each and analyze on 1% Seakem GTG Agarose TAE gel



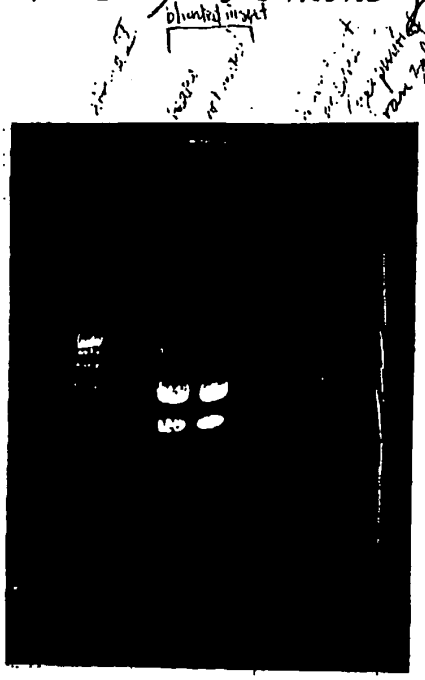
To the remainder add 10µl 3M NaAc + 250µl 100% EtOH
Store at -20°C for 15 min
Spin 10 min 4°C
Wash w/ 70% EtOH
Resuspend in TE (30µl)
Add 4µl 10X blue juice

To blunt ends, add 5 µl 10X T4 DNA Polymerase Buffer
+ 2 µl of dNTP mix (25mM) for final 1mM
+ 7 µl H_2O = 50 µl rxn ~~37° 30 min~~
+ 1 µl T4 DNA Polymerase 3U/µl 37° 30 min (11:30am - 12 noon)

Add 4 µl 0.25M EDTA
Add 55 µl. Phenol/Chloroform/Isoamyl Alcohol (25:24:1)
Shake, spin, save aq. layer

To aq. phase
Add 10
1/10 V 5M NaCl (= 5 µl)
Shake add 5 µl of 1X linear polyacryl
add 150 µl 100% EtOH
Chill @ -80°C 10'
Spin 10' 4°C
Wash pellet w/ 70% EtOH
Speedvac dry

Resuspend pellet in 36 µl TE
Run 2 µl on mini-gel

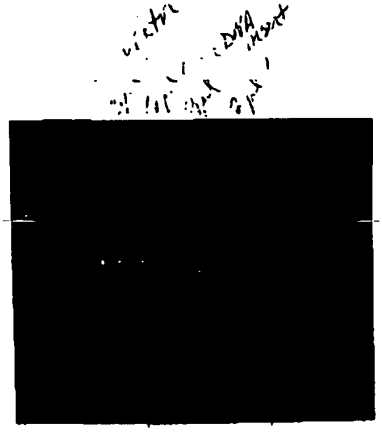


Add blue juice & run on 1% TAE Seaplaque agarose gel in cold room at 20 V (med strength app)
5:45pm - 11:00 am
Cut out insert (~2.3KB) & ligate into vector (see following page)

|X|6.5|

Ligation Exns

1) Run blunt vector + blunt insert on 1% GTG Seakem gel to quantitate relative amounts



Estimate that 3μl vector = 3μl cDNA
Want ~ twice as much insert as vector in 10μl rxn so use 4μl vector and 6μl insert

2) Heat tubes to 70°C for 10-15 min to melt agarose
3) Combine aliquots of melted gel slices in tubes pre-warmed to 70°C

tube	vector cut	vector not cut	blunt cDNA insert	H_2O	70°C 10 min	2X Ligation rxn mix (10 μl)
1	4μl	–	–	6μl	✓	10μl
2					✓	
3	4μl	–	6μl	4μl	✓	
4	–	4μl	6μl	6μl	✓	

2X Lig. rxn mix: 10μl 1M Tris Cl pH 7.5
1μl 1M $MgCl_2$
1μl 1M DTT
1.2μl 86 mM ATP
86.8μl H_2O
100μl

Take 35μl & add 4μl T4 DNA Ligase

Incubate 48 hr 12-16°C (3pm Sat - 7pm Mon)

X165 cont'd

Transformation of Ligations into MC1061/P3 E. coli

1. Melt the agarose/ligation mixtures at 70°C for 10-15 min.
2. Meanwhile thaw competent MC1061/P3 (from J. Ashkenas)

transform				Results #colonies
1	Lig #1	CIPed vector	5µl	56
2	Lig #2	insert (cDNA) only	5µl	28
3	Lig #3	CIPed vector + insert	5µl	30
4	Lig #4	UnCIPed vector	5µl	>500
5	TE only		5µl	47
6	pcDNA1 - 100ng/µl		1µl	>500

3. Add 5µl of melted agarose ligation mixtures or control alone. Mix by gentle shaking. Store on ice 30 min 12:45-1:15
4. Transfer to circulating water bath at 42°C. Incubate exactly 90 sec. DO NOT SHAKE TUBES.
5. Rapidly transfer to ice bath. Allow cells to chill 1-2 min.
6. Add 450µl LB medium warm to 37° & transfer to shaking incubator at 37°C. Incubate 1hr 1:25-2:25
7. Plate onto LB Amp/Tet (15µg/ml / 8µg/ml) plates 100µl/100mm plate. Incubate 37°C overnight

Results: Insert did not ligate into CIPed vector. Vector is CIPed well. Try blunting cDNA again and Geneclean both it and vector.

VII-6

Geneclean - vector + cDNA insert (#76)

	volume	2.5V NaI	glassmilk
insert -	150µl	375µl	10µl
vector -	150µl	375µl	10µl

Since ligation did not work between vector & cDNA, need to make sure ends of cDNA are blunt

Remove protruding 3' termini from cDNA insert

Combine: 15µl of genecleaned mSR-BI cDNA insert
1µl of 1:1:1:1 mix of dNTPs (25mM of each) ⇒ final 1.25mM
2µl of NEB Buffer 2 10X (final: 10mM Tris 10mM $MgCl_2$, 50mM NaCl 1mM DTT pH 7.9)
2.0 µl T4 DNA Polymerase (3 U/µl)
Incubate 15 min 12°C

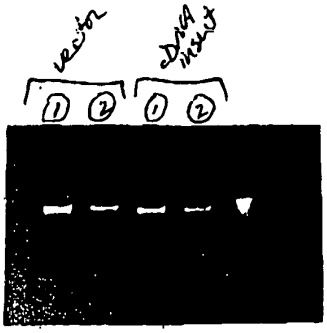
Inactivate polymerase by heating to 75°C for 10 min (11-11:10 am)

Run vector + cDNA insert on minigel to quantitate amts

1µl + 2µl TE ① → 1µl → + 2µl TE ②

run 2µl + 2µl 2x blue juice = 0.67

run 2µl + 2µl 2x blue juice = 0.22



For a 1 vector : 2 insert molar ratio use equal ng of vector & insert
Looks like vector stock = 1.5x cDNA

Ligate cDNA into vector

Ligation	blunt pcDNA1 CIPed	msR-B1 blunt cDNA	H_2O	BRL 5X T4 DNA Lig buffer	BRL T4 DNA Ligase (1U/μl)
1	3μl	-	4.5μl	2μl	0.5μl
2	3μl	4.5μl	-	2μl	0.5μl
3	1μl unCIPed vector	-	6.5μl	2μl	0.5μl

Mix well, spin & incubate 12-16°C (~16°C) 12:15pm - 10:30 Fri (~45hrs)

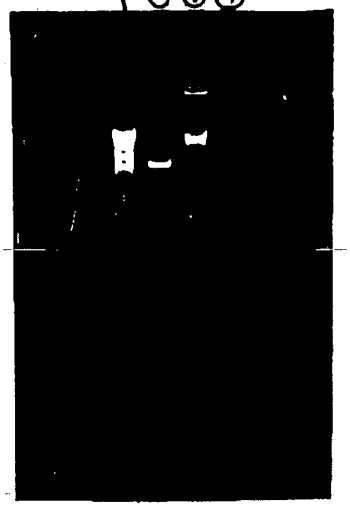
Transform MC1061/P3

transf		amt	results # colonies
1	Lig #1	5μl	123
2	Lig #2	5μl	32
3	Lig #3	5μl	33
4	TE only	5μl	
5	pcDNA1 388ng/μl 4μl	4μl	>1000

transformation worked but other results don't make sense

1) Add 5μl of above DNA to thawed competent MC1061/P3. Mix by gentle flicking Store 30' ice (10:40-11:10)
2) Transfer to circulating water bath at 42°C. Incubate exactly 90 sec.
3) Rapidly transfer to ice bath. Allow cells to chill 1-2 min.
   Add 450μl LB Warm to 37°C & transfer to shaking incubator at 37°C.
4) Plate onto LB Amp/Tet (150μg/ml / 8μg/ml) 100μl/100mm dish Incubate 37°C O/N.

Ran ligation rxns on gel to check



Lig

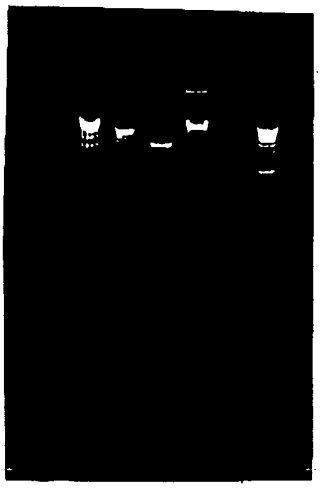
#1 - one piece but 6Kb!? Should be 4Kb. Must have cut the wrong vector? Redo preparation of blunt-cut c1pcd pcDNA1 (see below)

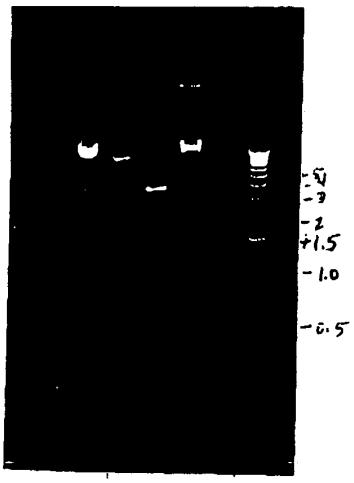
#2 - some ligations appear to have worked possibly cDNA - cDNA worked best. Next time cut way back on amt of cDNA : vector.

#3 - can't see any DNA

Run with 1 Kb standards

Results:
blunt cut pcDNA1 is 4Kb as it should be
λHindIII stds are junk





X167

Redo Ligations

Ligation	blunt pcDNA1 CIPed	mSR-BI blunt cDNA	H_2O	BRL 5X T4 DNA Lig buffer	BRL T4 DNA Ligase (1U/μl)
1	1 μl	–	14	4 μl	1 μl
2	1 μl	1 μl	13	4	1
3	1 μl	1 μl of 1:5	13	4	1
4	1 μl of 1:5	1 μl of 1:5	13	4	1
5	1 μl of 1:5	–	14	4	1

Mix well, spin & incubate 12-16°C (submerged) O.N. (5pm - 2pm)

Transform				Results # colonies
1	Lig #1	5 μl	→ 80 μl comp MC1061/P3 cells	68
2	2	5 μl	↓	136
3	3	5 μl	↓	130
4	4	5 μl	↓	65
5	5	5 μl	↓	19 + ~30 in one large group
6	TE	5 μl	↓	5
7	pcDNA1	1 μl	↓	>2000

On ice 30' 2:30 - 3pm
Shock 42°C 90 sec
On ice 1-2 min
Add 400 μl LB & shake 37°C 45'
Plate onto LB A/T (#1-4 my plates, # 5-7 Xxxxxx's plates)
100 μl

PCR colonies

Purpose: Determine if colonies from transformation plate #2 contain mAR-BI in correct orientation

Set A (determine if insert present): T7 + ST6 primers
Set B (determine orientation): T7 + OSA4.4B

For 55 rxns of 20µl each

110 µl 10X PCR buffer (QG)
27.5 µl T7 primer @ 1 pmol/µl (VH)
425.7 µl H_2O
2.2 µl dATP
2.2 µl dCTP
2.2 µl dGTP
2.2 µl dTTP
5.5 µl Taq polymerase

(A) 412.5 µl
+ 137.5 µl ST6 @ 1 pmol/µl

412.5 µl (B)
+ 2 µl stock OSA4.4B (unknown conc.)
+ 135.5 µl H_2O

Pick 20 colonies
#21 neg control colony from transformation plate #1
#22 control #36 pure plasmid in SN BI in pcDNA1 backward

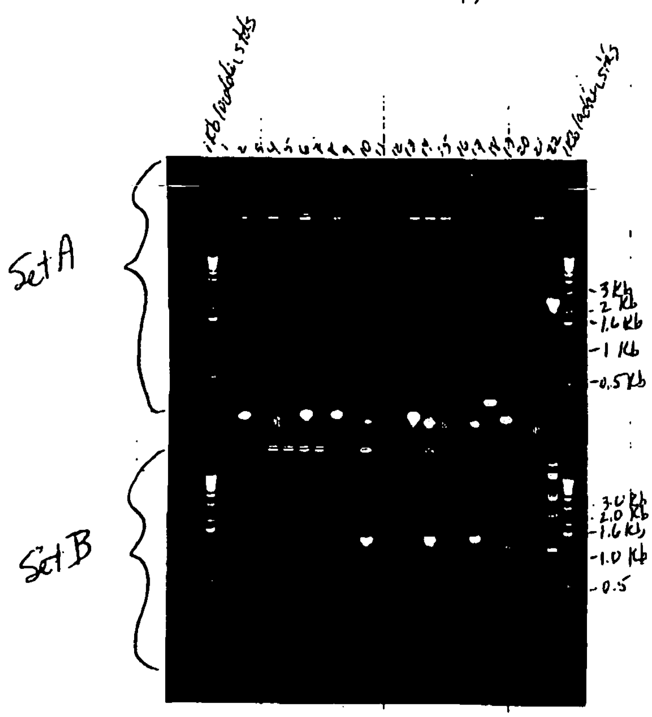
From 20 random colonies

10 will probably be vector only (no insert) - background
10 will " contain cDNA
of which 5 will be backwards
and (5 will be correct orientation

[redacted]

Run gel on PCR products

Run 1% GTG-Agarose. 1X TAE



Results: Only the positive control pure plasmid #76 (pcDNA1/neo mSRBI in backward) worked with the T7 + SP6 primers

However, a band of ~1.3 Kb was present in #10, #14 + #17. The expected size is ~1.35 Kb for mSR BI in correct orientation. Hopefully these are real.

Plasmid midiprep

preps: #2, #10, #14, #17 colonies from ligation/transform plate 2

Day 1

1. Pick a single colony into 25 mls LB Amp/Tet and grow overnight shaking 37°C.

Day 2

2. Take 400 µl, put into freezer vial, add 100 µl glycerol and freeze at -150C.
3. Transfer half to Falcon 2059 15 ml tube on ice.
4. Spin in SS-34, 9000 rpm, 2 min 4C. Discard supe, add remaining bacteria and respin.
5. Dry pellet as much as possible.
6. Resuspend pellet in 500 µl ice cold solution I by vigorous vortexing.
7. Add 1 ml fresh solution II (0.2 N NaOH, 1% SDS)
   for 10 ml: 1 ml 2 N NaOH
   0.5 ml 20% SDS
   8.5 mls ddH20
   Swirl gently until clear. Do not vortex. Leave on ice 10 min.
8. Add750 µl solution III (ice-cold). Close tube and mix contents by shaking vigorously several times. Store on ice 5'. A flocculent white precipitate should form.
9. Centrifuge 15', 4°C, 9000 rpm.
10. Recover supe and add equal volume of phenol:chloroform. Mix by vortexing.
11. Spin 9000 rpm, 5'. Recover aq. phase.
12. Add 2 volumes, ethanol r.t., vortex, let stand 5'.
    Spin in SS-34 rotor for 15' 9,000 rpm.
13. Carefully aspirate off all liquid (want it dry) and rinse once in cold 70% EtOH. Aspirate again getting any extraneous drops. Airdry 10' minutes.
14. Redissolve in 100 µl TE plus DNAse-free RNAse (20 µg/ml). Vortex briefly. Incubate 37C (0.5 - 2 hr. Transfer to ~~sterile~~ eppendorf.

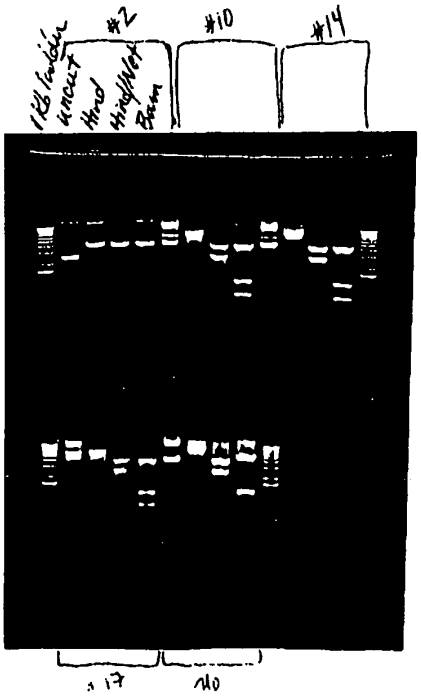
~~15. Quantitate by dilutions onto EtBr plate.~~

Digests

Digest	DNA	10X Acc65I 2µl	2µl	ACC65I BstEII 10X 2µl	H_2O 14µl	+ enzyme 2
1						
2						
#4						
5	colony 10					
6						
7						
8						
9	colony 14					
10						
11						
12						
13	colony 17					
14						
15						
16						
17	#76 MSP BJ (backwards)					
18						
19						
20						

Incubate @ 37°C
12:45 - 3:45

Run on 1% Seakem GTG TAE gel



Results: Neg control #2 looks like pCRVA1 only. #10 #14 #17 all look same with insert size ~2.5 Kb. Not identical to #76 MSP BJ (backwards). Everything looks good! I think all three #10, #14, #17 are all in correct direction.
~~See next page~~

X168

DEAE dextran transfections of COS M6 cells

materials:

1. 35 mm dishes
2. DMEM with 10% FBS
3. Chloroquine (40 mM in CMF PBS, sterile filtered)
4. DNA
5. CMF PBS
6. DEAE-dextran (10 mg/ml in CMF PBS (autoclaved))
7. DMSO
8. cPBS
9. sterile tips

method:

day 0 (set up cells)

Set COS M6 cells in 100 mm dishes

(Set 1 confluent T75 into 2.25 100 mm dishes; or 1.5 x 10^6 cells/dish in 10 ml DMEM with 10% FBS

day 1 (transfect)

1. In sterile polypropylene tubes prepare for each dish add (in order):
   a) add CMF PBS to 1.9 ml
   b) DNA - 5 µg/dish
   c) 100 µl of 10 mg/ml DEAE-dextran, vortex well (7 seconds)

tube	# plates	DNA			CMF PBS	10 mg/ml DEAE-dextran
1	1	pcDNAI (1.29 mg/ml)	15µg	3.9ml	1.9 ml	100µl
2	3	pmSR-BI (2.10 mg/ml)		30µl	5.7ml	300µl
3						
4						

2. Rinse cells with 10 mls CMF PBS (37C). Aspirate PBS.
3. Add transfection cocktail from step 1 and distribute evenly. Incubate 30 minutes, distributing liquid every 10 min. 1:30 - 2:00
4. Add 8 ml DMEM 10% FBS + 80 µM chloroquine and incubate 37C 2.5 hrs. 4:30
5. Aspirate off medium and replace with 5 ml 10% DMSO in DMEM 10% FBS for 2.5 min.
6. Aspirate off and wash once with 10 mls cPBS.
7. Refeed with 10 ml warm DMEM 10% FBS/dish. Incubate overnight.

Lipoprotein 4°C Binding Assay

materials:
1. 6-well dishes
2. DMEM with 10% FBS
3. iodinated and unlabeled lipoprotein
4. Tris-HCl wash buffer
5. Tris-HCl BSA wash buffer
6. 0.1 N NaOH
7. 10 x 75 tubes

method:

day 0: Set up cells

Set transfected COS in 6-well dishes at 1 x 10^6 cells/well in 3 ml/well DMEM + 10% FBS + 1 mM Nabutyrate.

day 1: Binding assay

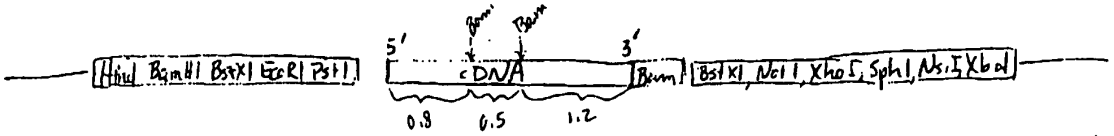
1. Cool cells down on ice for 30 minutes.
2. Refeed cells labeled ligand 1 ml/well (Hams + HEPES + ~~10% FBS~~ 5% NCLPDS)
   2 Hot - ^{125}I-lipoprotein: 10 mg/ml ^{125}I AcLDL #62 (0.55 mg/ml)
   ~~1 Hot + cold~~: competitors (a) 400 mg/ml
   Incubate 2 hrs 4°C (in cold room) on shaker.
3. Wash cells (1 ml each)
   3X fast with ~~BSA~~ wash buffer
   ~~2X 5 min with BSA wash buffer~~
   ~~2X fast with Tris wash buffer~~
4. Add 1.0 ml 0.1 N NaOH. Leave 20 min r.t. on shaker
5. Remove 50 µl to 10 x 75 tube and freeze for Lowry.
6. Count 500 µl of rest of sample.
7. Count 10 µl of medium + label for specific activity.

AcLDL #55 4.6 mg/ml
LDL #16B 39
HDL #3 31.2
VLDL #5 20.7

309 µl ^{125}I-AcLDL #62 + 17 mls Hams + 5% NCLPDS + 10 mM Hepes

4 mls + 348 µl AcLDL #55
2 mls + 34.5 µl LDL #16B
2 mls + 25.6 µl HDL #3
2 mls + 38.6 µl VLDL #3

mSR-BI in correct orientation:



mSR-BI (#76) in incorrect orientation: